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Cofactor-Interaction Motifs and the Cooption of a Homeotic Hox Protein into the Segmentation Pathway of *Drosophila melanogaster*

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Summary

Some *Drosophila Hox*-complex members, including the segmentation gene *fushi tarazu* (*Dm-ftz*), have nonhomeotic functions [1]. Characteristic expression in other arthropods supports an ancestral homeotic role for *ftz* [2, 3], indicating that *ftz* function changed during arthropod evolution. Dm-Ftz segmentation function depends on interaction with *ftz*-F1 [4–6] via an LXXLL motif [7–9] and homeodomain N-terminal arm [7]. Hox proteins interact with the cofactor Extradenticle (Exd) via their YPWM motif [10–14]. Previously, we found that Dm-*ftz* mediates segmentation but not homeosis [14], whereas orthologs from grasshopper (Sg-*ftz*) [15] and beetle (Tc-Ftz) [16], both containing a YPWM motif, have homeotic function. Tc-Ftz, which unlike Sg-Ftz contains an LXXLL motif, displays stronger segmentation function than Sg-Ftz [14, 17]. Cofactor-interaction motifs were mutated in Dm-Ftz and Tc-Ftz and effects were evaluated in *Drosophila* to assess how these motifs contributed to Ftz evolution. Addition of YPWM to Dm-Ftz confers weak homeotic function, which is increased by simultaneous LXXLL mutation. LXXLL is required for strong segmentation function, which is unimpeded by the YPWM, suggesting that acquisition of LXXLL specialized Ftz for segmentation. Strengthening the Ftz/Ftz-F1 interaction led to degeneration of the YPWM and loss of homeotic activity. Thus, small changes in protein sequence can result in a qualitative switch in function during evolution.

Results and Discussion

Key motifs were altered in both Dm-Ftz and Tc-Ftz to test how changes in cofactor interaction-motifs contributed to the functional evolution of Ftz proteins (see Figure S1 in the Supplemental Data available with this article online). The homeodomain N-terminal arm, which confers functional specificity of homeotic Hox proteins [18–20] and has been suggested to contribute to Dm-Ftz/Ftz-F1 interactions [7], was mutated from KRTRQTYTR to that of Dm-Scr—KRQRTSYTR (Dm-FtzNTS and Tc-FtzNTS; Figure S1)—to assess its importance for segmentation function. Dm-Ftz does not contain a YPWM motif but rather the sequence FNWS upstream of its homeodomain, at a position similar to the YPWM motif in homeotic Hox proteins (Figure S1). The FNWS of Dm-Ftz was changed to YPWM (Dm-FtzYPWM) to determine whether the YPWM motif is sufficient to confer homeotic function and to assess whether the presence of this Exd-interaction motif interferes with segmentation function. In a reciprocal construct, the YPWM motif of Tc-Ftz was changed to FNWS (Tc-FtzFNWS). This protein retains the W residue important for YPWM-Exd interaction [12, 13]. The motif was mutated to AAAA in the construct Tc-FtzAAAA to test the ability of Tc-Ftz to mediate homeotic functions without YPWM-mediated Exd interactions because it has been shown that direct Hox/Exd interaction mediated by the YPWM motif is abolished by mutation of YPWM to alanines [21–23]. The LRALL sequence in Dm-Ftz binds directly to the AF-2 domain of the orphan nuclear receptor Ftz-F1 [7, 8] and corresponds to an LXXLL motif found in nuclear-receptor coactivators [24]. Substitution of the conserved leucines to alanines abolishes interaction with nuclear hormone receptors [24], and mutation of LRALL to LRAAA in Dm-Ftz abolished interaction with the Ftz-F1 AF-2 domain [8]. This motif was changed to LRAAA (Dm-FtzLRAAA) to assess the importance of the Dm-Ftz LRALL motif for segmentation and to establish whether its presence masks homeotic potential. Additionally, the LRALL loss-of-function mutation was combined with the YPWM insertion or N-terminal arm substitution (Dm-FtzLRAAA/YPWM; Dm-FtzLRAAA/NTS). A myc-tag was added to the N terminus of each protein to compare protein levels between different transgenic lines (Supplemental Data). Constructs were placed under the control of an upstream activator sequence (UAS) [25] or a heat-inducible promoter.

The effect of cofactor-interaction motifs on Dm-Ftz segmentation function was tested by expressing altered Dm-Ftz proteins at 2 hr after egg laying (AEL) with the heat-shock system. Ectopic expression of Dm-Ftz in embryos at this time causes an "anti-*ftz*" phenotype characterized by the development of only half the segments, with denticle belts corresponding to segments T2, A1, A3, A5, and A7, visible in larval cuticles [26]. Cuticles were scored as "wild-type" (no effect on the cuticle), "partial anti-*ftz*" (not all denticle belts affected in an anti-*ftz* manner), "complete anti-*ftz*" (only T2, A1, A3, A5, and A7 denticle belts present; see Figures S2A–S2C), and "other" (including non-anti-*ftz* pairing defects and random cuticular phenotypes).

Substitution of the N-terminal arm of the Ftz homeodomain had a slight inhibitory effect on segmentation function: Dm-FtzNTS induced complete anti-*ftz* phenotypes in fewer embryos than Dm-Ftz (~27% versus ~42%; Figure S2D). The insertion of the YPWM motif had no significant effect on Dm-Ftz segmentation function. Both Dm-Ftz and Dm-FtzYPWM induced complete and partial anti-*ftz* phenotypes similarly (Dm-Ftz: partial ~27%, complete ~42%; Dm-FtzYPWM: partial ~31%,

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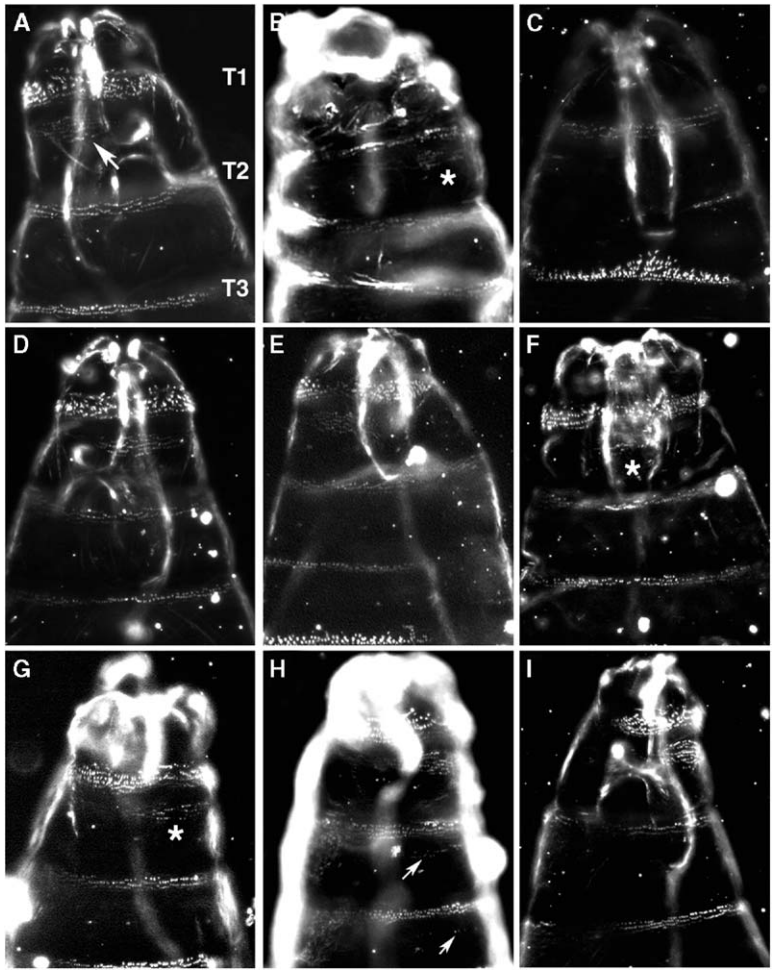

Figure 1. The YPWM Motif Is Necessary but Not Sufficient for Homeotic Function during Embryogenesis

Mutated Ftz proteins were expressed with the *arm*-GAL4 driver. Cuticles were assessed for homeotic transformations.

(A) Ectopic expression of the myc-tag had no effect on cuticle development. T1, T2, and T3 segments and characteristic T1 beard (arrow) are indicated.

(B) Expression of Antp caused head-involution defects and transformation of the T1 segment toward T2, marked by the reduction of the T1 beard (asterisk). Denticles of the T1 segment have taken on T2 morphology.

(C) Dm-Ftz caused segmentation defects but no discernable homeotic transformations. Here, head morphology is normal, T1 has merged with T2, and T3 has partially fused with A1.

(D) Expression of Dm-FtzLRAAA had no effect on cuticle formation.

(E) Dm-FtzYPWM did not cause discernable homeotic phenotypes. Here, head involution was slightly disrupted, but the thoracic segments formed normally.

(F) Expression of Dm-FtzLRAAA/YPWM caused an Antp-like homeotic transformation of the T1 segment. Head involution failed, and the T1 segment has taken on T2 identity, marked by the reduced T1 beard (asterisk).

(G) Expression of Tc-Ftz caused head-involution defects and the transformation of the T1 segment toward T2, observed as the reduction of the T1 beard (asterisk).

(H) Ectopic expression of Tc-FtzNTS caused Scr-like gain-of-function phenotypes [28]. Head involution failed, and the T2 and T3 segments have taken on T1 identity, indicated by ectopic T1 beard bristles in both T2 and T3 (arrows).

(I) Neither ectopic expression of Tc-FtzAAAA nor that of Tc-FtzFNWS (data not shown) had an effect on cuticle formation, demonstrating that the YPWM motif is necessary for homeotic function in this assay. Homeotic effects observed in (F)–(H) were directly mediated by ectopic protein because no ectopic Antp or Scr expression was observed in the presence of Tc-Ftz and Dm-FtzLRAAA/YPWM or Tc-FtzNTS, respectively (data not shown).

complete ~40%). This suggests that the presence of the YPWM motif in an evolving Ftz protein containing an LXXLL motif would not have hampered its ability to function in segmentation pathways. Comparing the sum of cuticles displaying partial or complete anti-*ftz* phenotypes, all three proteins, Dm-Ftz, Dm-FtzYPWM, and Dm-FtzNTS, behaved similarly (~70% for Dm-Ftz, ~62% for Dm-FtzNTS, and ~72% for Dm-FtzYPWM), indicating that the overall ability of Dm-Ftz to function in segmentation is only weakly affected by the N-terminal arm of the homeodomain.

As expected from previous reports [7–9], mutation of LRALL significantly impaired segmentation function: Dm-FtzLRAAA never induced the complete anti-*ftz* phenotype, although partial phenotypes were observed (~44%). Thus, acquisition of the LXXLL motif can account to a large extent for the switch in Ftz function during evolution. However, in the absence of a functional LXXLL motif, additional contributions of other motifs to segmentation potential were uncovered. Switching the N-terminal arm of the homeodomain in the context of the Dm-FtzLRAAA mutation additionally hampered segmentation potential (compare Dm-FtzLRAAA/NTS partial anti-*ftz*, ~25%, to Dm-FtzLRAAA, ~44%). This is consistent with a weak contribution of the Ftz-specific N-terminal arm to segmentation. Thus, the N-terminal arm may have contributed to segmentation potential of ancestral Ftz proteins, which did not contain LRALL, making them more competent than other Hox proteins to interact with Ftz-F1 and therefore predisposing them for segmentation function. Dm-FtzLRAAA/YPWM induced only partial anti-*ftz* phenotypes in ~25% of embryos, suggesting that the presence of the YPWM motif can inhibit segmentation function when a strong Ftz-F1 interaction motif is absent. Thus, although the YPWM motif may have hindered segmentation potential of Hox proteins lacking an LXXLL motif, this inhibition was overcome in Ftz by the LRALL acquisition.

The effect of cofactor-interaction motifs on the homeotic functions of Ftz proteins was assessed during embryogenesis, when homeotic Hox proteins are active, by expression with an *arm*-GAL4 driver. As for the wild-

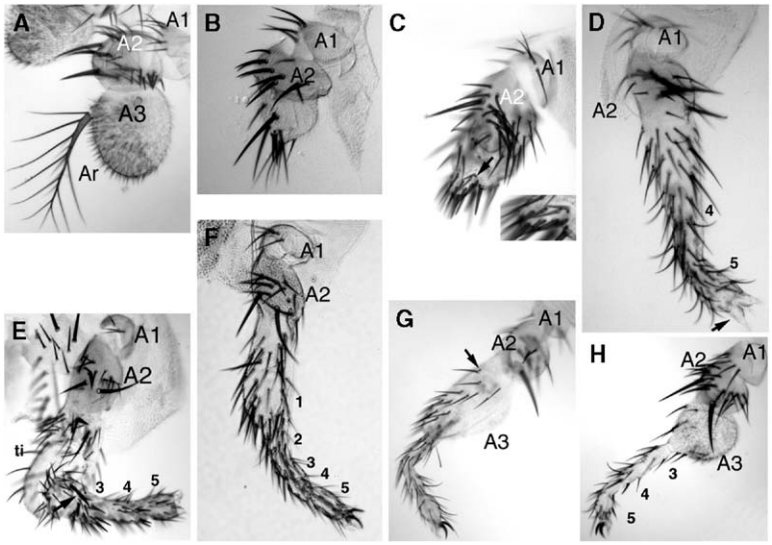

Figure 2. The YPWM Is Sufficient but Not Necessary for Antenna-to-Leg Transformations

Ftz proteins were expressed throughout development in the antennal disc with the *Dll*GAL4 driver. Antennae were evaluated for transformation toward leg identity.

(A) Expression of myc-tag alone had no effect on antennal morphology: Arista (Ar) and all antennal segments (A1–A3) developed normally.

(B) Expression of Dm-Ftz caused the deletion of the arista and the truncation of the A3 segment.

(C) Dm-FtzLRAAA caused a similar phenotype. However, some ectopic bristles on the A3 segment were associated with bracts (arrow), a characteristic of leg bristles. Inset shows a magnified view of the bracted bristles.

(D) Dm-FtzYPWM caused weak antenna-to-leg transformations. The arista and the A3 segment were transformed into two tarsal segments (4 and 5). A claw was not formed, but the sensory pad associated with the claw was observed (arrow).

(E) Expression of Dm-FtzLRAAA/YPWM caused a strong antenna-to-leg transformation. The ectopic limb encompassed the tarsal segments and the distal tibia (ti), including an apical bristle (arrow), a characteristic of the T2 leg.

(F) Tc-Ftz caused the transformation of the arista and A3 segment to complete tarsus. Five tarsal segments (1–5) and claw were formed.

(G) Tc-FtzFNWS caused weaker homeotic transformations. The arista was transformed into three tarsal segments (3–5). The A3 segment was not completely transformed, but ectopic bracted bristles (arrow) were observed, indicating a mild transformation toward leg identity.

(H) Expression of Tc-FtzAAAA caused homeotic transformations. The arista was transformed into three tarsal segments (3–5), but no bracted bristles were observed on A3. Effects observed in the presence of ectopic Dm- and Tc-Ftz proteins were direct because ectopic Antp was not observed in these discs (data not shown).

type, the head and three thorax segments, T1, T2, and T3, of embryos expressing only myc-tag developed normally, and the characteristic T1 beard was clearly visible (Figure 1A, arrow). Ectopic expression of Antp caused head-involution failure and transformation of T1 toward T2, marked by reduction of the T1 beard ([27]; Figure 1B, asterisk). No homeotic effects were induced by ectopic Dm-Ftz, but partial anti-*ftz* phenotypes were observed (Figure 1C). Dm-FtzLRAAA was expressed to test whether a strong interaction with Ftz-F1 masks homeotic functions of Dm-Ftz. No effect on development was observed (Figure 1D), indicating that the protein lacks strong inherent homeotic potential. Although Dm-Ftz interacts with Exd in glutathione S-transferase (GST) pull-down assays (Figure S3), this binding is apparently not sufficient for functional interaction between Exd and Dm-Ftz in vivo. Dm-FtzYPWM was expressed to test whether addition of the YPWM motif is sufficient to confer homeotic activity. No obvious homeotic effects were observed: Partial anti-*ftz* phenotypes (not shown) and slight head-involution defects were detected (Figure 1E). This indicates that insertion of a strong Exd-interaction motif was not sufficient to switch the function of Dm-Ftz from segmentation to homeosis in the embryo. In contrast, clear homeotic effects were observed when Dm-FtzLRAAA/YPWM was expressed. Head involution failed, and the T1 segment was transformed toward T2 identity, indicated by reduction of the T1 beard (Figure 1F). Thus, a strong Antp-like homeotic function of Dm-Ftz was only observed when strong interactions with Ftz-F1 were abolished, and the YPWM motif was inserted. Lastly, changing the homeodomain N-terminal arm to that of Scr was not sufficient to confer homeotic function: Neither Dm-FtzNTS nor Dm-FtzLRAAA/NTS caused homeotic phenotypes (not shown).

Ectopic expression of Tc-Ftz with the *arm*-GAL4 driver caused Antp-like homeotic transformations (Figure 1G) similar to those observed previously when expression was induced at 5.5 hr AEL by heat shock [14]. Substitution of the Tc-Ftz homeodomain N-terminal arm for that of Scr caused the T2 and T3 segments to take on T1 identity, marked by formation of ectopic T1 beard bristles (Figure 1H; arrows). Similar transformations were observed in the presence of ectopic Scr [28], indicating that Tc-FtzNTS functions in an Scr-like fashion. Thus, the homeodomain N-terminal arm confers specificity of homeotic function [18–20] but not homeotic function as such. In contrast, the YPWM motif was necessary for homeotic activity per se: Neither Tc-FtzFNWS nor Tc-FtzAAAA induced homeotic transformations (data not shown and Figure 1I, respectively).

A hallmark of the central homeotic genes *Scr*, *Antp*, and *Ubx* is their ability to transform antenna toward leg identity upon misexpression in the antennal primordia [28–30]. Altered Ftz proteins were expressed in a sustained fashion in the distal portion of developing imaginal discs with the *Dll*-GAL4 driver [31] to test the effect of cofactor-interaction motifs on the ability of Ftz proteins to induce antenna to leg transformations. As previously reported, ectopic expression of Dm-Ftz in this system resulted in deletion of the arista and malformation of the A3 segment (Figure 2B; compare with Figure 2A), the deletion caused by massive cell death in the region of ectopic protein expression [14]. A similar effect was observed with Dm-FtzLRAAA (Figure 2C). Interestingly, some ectopic bristles on the malformed A3 segment were associated with bracts (Figure 2C, arrow;

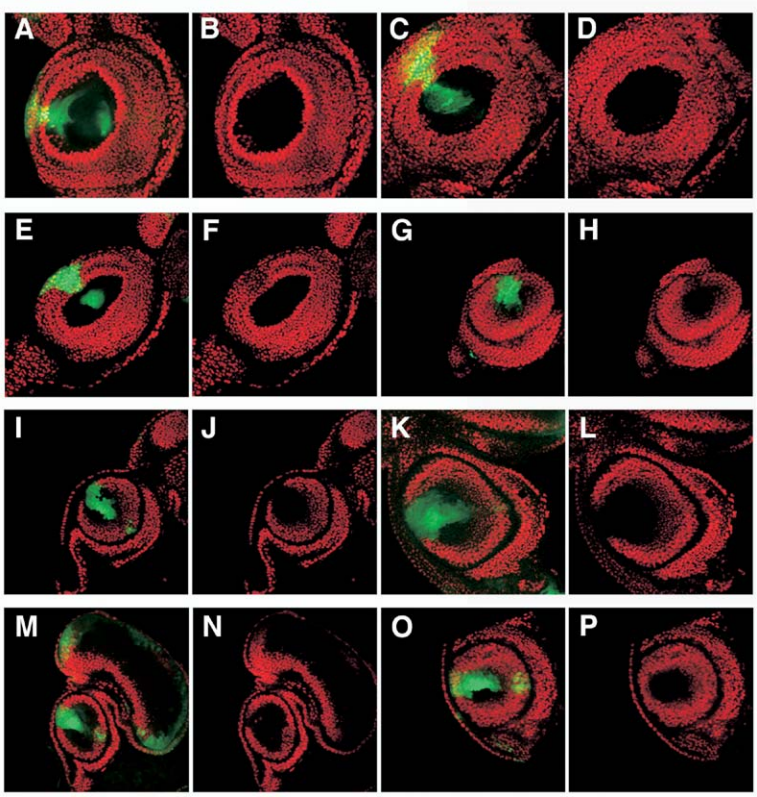

Figure 3. The YPWM Is Necessary for Repression of Hth

To test the ability of Ftz proteins to act in a homeotic fashion at the molecular level, we expressed Ftz proteins with a *dpp*-GAL4 driver coupled to UAS-GFP and assessed the ability to repress Hth. The area in which ectopic proteins are expressed is marked by GFP.

(A and B) Expression of the myc-tag had no effect on Hth expression (red). Expression remained uniform and overlapped with GFP (green; overlap in yellow).

(C and D) Dm-Ftz also had no effect on Hth expression. Note the clear overlap between GFP and Hth (C) and the uniform expression pattern (D).

(E and F) Dm-FtzLRAAA had a weak effect on Hth expression. Although an overlap between GFP and Hth was still observed (E), the level of Hth in the area expressing ectopic protein was lower than in the rest of the disc (F).

(G and H) A similar result was obtained with Dm-FtzYPWM. Hth was not completely repressed, but the level of expression in the *dpp* domain was weaker (H).

(I and J) Dm-FtzLRAAA/YPWM completely repressed Hth. An overlap between GFP and Hth was not observed (I), and Hth expression was no longer uniform (J). This resembles the effect observed in the presence of ectopic Tc-Ftz (K and L).

(M and N) Tc-FtzFNWS partially repressed Hth. An overlap between GFP and Hth was still detected (M), but the level of Hth expression was lower in the regions expressing ectopic Tc-FtzFNWS (N).

(O and P) Tc-FtzAAAA had no effect on Hth expression. Hth expression remained uniform, and no decrease in expression levels in the *dpp* domain was observed.

inset), a characteristic of leg bristles. Therefore, in the antennal tissue, subtle homeotic effects can be observed when the LXXLL motif is nonfunctional. In contrast to the assay in embryos, insertion of the YPWM motif into Dm-Ftz imparted homeotic potential here. Dm-FtzYPWM transformed the arista and A3 segment into the two distal-most tarsal segments (Figure 2D).

Expression of Dm-FtzLRAAA/YPWM led to a very strong transformation of the arista and A3 segment toward leg identity; the arista was completely transformed toward tarsus identity (Figure 2E). Tarsal segments 3–5 and the sensory pad were clearly visible. Proximal to the third tarsal segment, an apical bristle, a distinguishing characteristic of the T2 leg, was observed (Figure 2E, arrow). Comparison of phenotypes caused by Dm-FtzYPWM and Dm-FtzLRAAA/YPWM suggests that strong interaction with Ftz-F1 can inhibit the homeotic potential of Ftz proteins, even in the presence of the YPWM motif.

Expression of Tc-Ftz caused complete transformation of the arista and A3 segment toward tarsal identity, observed as formation of five tarsal segments, including claw ([14]; Figure 2F). Expression of Tc-FtzFNWS caused slightly weaker antenna-to-leg transformations: Only the three most-distal tarsal segments, including claw, were formed (Figure 2G). The presence of bracted bristles on the A3 segment (arrow) indicated that this segment was also weakly transformed toward leg identity. Note that the "W" conserved in Dm-Ftz FNWS and important for Hox-YPWM/Exd interaction [12, 13] and for Ftz binding to Exd (Figure S3) is not sufficient for strong homeotic function. Homeotic function of Tc-FtzAAAA was even weaker but was still observed: The arista was completely transformed into tarsal segments, including claw, but ectopic bristles on A3 were not bracted (Figure 2H). Thus, the YPWM motif is sufficient to confer homeotic function in the context of Dm-Ftz, but it is not necessary for homeotic arista transformation generated by Tc-Ftz. This suggests the presence of additional, unidentified Tc-Ftz motifs that are able to confer weak homeotic function, possibly via interaction with Exd (see also [32–34]).

Homeotic Hox proteins cause antenna-to-leg transformations, at least in part, by repressing Homothorax (Hth) expression in antennal discs [35]. Ftz proteins were expressed with a *dpp*-GAL4 driver coupled to UAS-GFP, marking expressing cells, to test their effect on Hth expression. Hth is normally expressed uniformly in the antennal disc, excluding only the distal-most region (Figures 3A and 3B; [36]). Dm-Ftz had no effect on Hth expression [14]. Hth was uniform (Figure 3D), and clear overlap between Hth staining (red) and GFP expression (green) was observed marking cells expressing both Dm-Ftz and Hth (Figure 3C; yellow). Slight reduction of Hth expression was observed with Dm-FtzLRAAA: The area in which ectopic Dm-FtzLRAAA

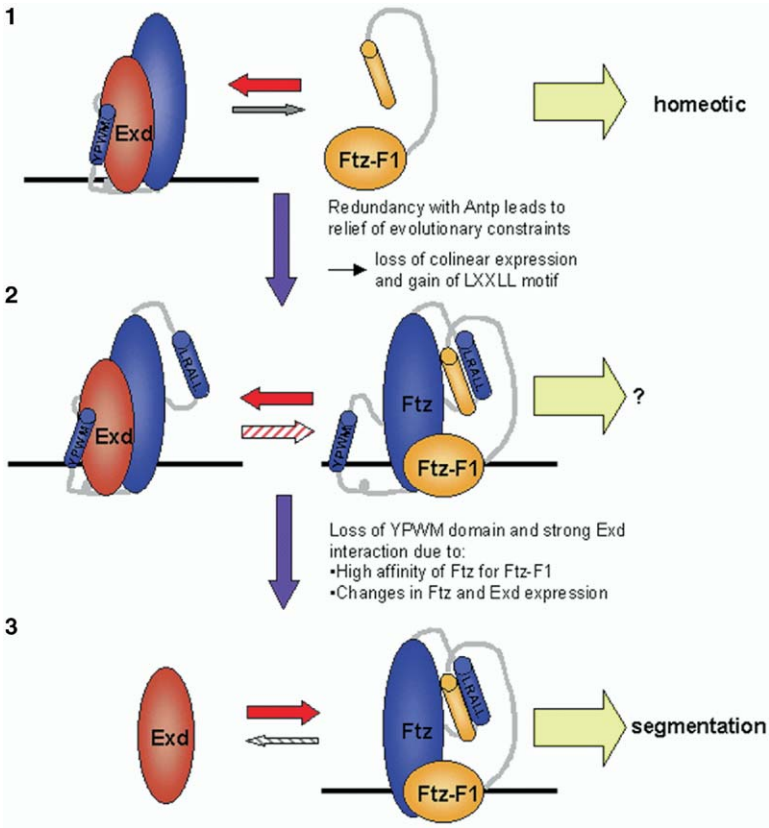

Figure 4. Model for the Functional Evolution of Ftz

The functional evolution of Ftz is proposed to have taken place in three major steps. (1) Expression data from chelicerates [2] and myriapods [3] suggest that ancestral Ftz had homeotic function (yellow arrow). These Ftz proteins probably interacted strongly with Exd (red arrow), but through the homeodomain N-terminal arm, they also had the potential to interact with Ftz-F1 (gray arrow). Functional redundancy with Antp led to the release of evolutionary constraints on Ftz protein and *ftz* regulatory regions (purple arrow). (2) Acquisition of the LRALL motif allowed stronger Ftz/Ftz-F1 interactions (pink arrow), whereas the presence of the YPWM domain also allowed strong interactions with Exd. The function of such an "intermediate" Ftz (such as Tc-Ftz) is unclear ([41]; yellow arrow). Strengthening of the Ftz/Ftz-1 interaction may have led to higher affinity of Ftz for Ftz-F1 than for Exd. Also, Ftz- and Exd-expression changes may have occurred that prevented the two proteins from interacting, together leading to the loss of the YPWM motif in Ftz and, thereby, loss of functional interaction with Exd (purple arrow). (3) Dm-Ftz has lost the ability to functionally interact with Exd, which is most likely due to the degeneration of the YPWM motif. An interaction is only detectable in vitro (gray arrow). Dm-Ftz has very high affinity for Ftz-F1 (red arrow) and functions solely in segmentation (yellow arrow).

was expressed only weakly stained for Hth (Figures 3E and 3F). Similarly, Hth staining was also reduced, although not totally absent, where Dm-FtzYPWM was expressed (Figures 3G and 3H). Thus, the weak antenna-to-leg transformations induced by these proteins correlated with the ability to only weakly repress Hth. Expression of Dm-FtzLRAAA/YPWM caused an almost complete repression of Hth, such that no overlap between GFP and Hth was observed, and Hth expression was no longer uniform (Figures 3I and 3J). Together, these results demonstrate that addition of the YPWM motif to Dm-Ftz is sufficient to confer weak homeotic potential but that simultaneous loss of the LXXLL motif is necessary for strong homeotic function. This further suggests that the acquisition of LRALL by Ftz during evolution would have weakened homeotic activity.

Ectopic expression of Tc-Ftz caused repression of Hth ([14]; Figures 3K and 3L): Hth expression was no longer uniform, and overlap between Hth staining and GFP was not observed. Tc-FtzFNWS had a slightly weaker effect on Hth expression. Only a weak overlap between GFP expression and Hth staining was observed (Figure 3M), and although the area of Hth expression was still uniform, Hth expression was reduced where Tc-FtzFNWS was expressed (Figure 3N). Tc-FtzAAAA had no effect on Hth. An overlap between Hth and GFP was clearly visible (Figure 3O), and Hth expression was uniform (Figure 3P). Thus, the YPWM motif is necessary for complete repression of Hth in the antennal disc by Tc-Ftz, and this, in turn, is required for strong antenna-to-leg transformation.

Conclusions

We have demonstrated that the "devolution" of Dm-Ftz from a protein with segmentation function to one with homeotic function can be achieved by changing only 5 amino acids (LRAAA and YPWM). In contrast, as proposed in Figure 4, the actual functional evolution of Ftz from a homeotic protein to one with exclusive segmentation function took several million years and involved complex changes in the expression of *ftz* and presumably other homeotic *Hox* genes. Our results indicate that acquisition of the LXXLL motif allowed stronger interaction between Ftz and Ftz-F1, although this in itself did not abolish homeotic function (see results with Tc-Ftz). However, the strengthening of the Ftz/Ftz-F1 interaction over time may have made a Ftz/Exd interaction nonfunctional (see results with Dm-FtzYPWM). This in turn may have led to a relief of evolutionary constraints on the YPWM motif and ultimately to loss of homeotic function.

In arthropods, the emergence of evolutionary novelties has been correlated with shifts in the expression of *Hox* genes along the anterior posterior axis [37, 38], accessibility of Hox target genes [32], and changes in the coding region of *Hox* genes [14, 39, 40]. Here, we have presented evidence that the cooption of a Hox protein with presumably ancestral homeotic function

into the segmentation pathway of *Drosophila* was due not only to drastic changes within its regulatory region, but also to simultaneous, significant changes in the amino acid sequence of key cofactor-interaction motifs. It is an intriguing possibility that the recruitment of Ftz into the segmentation pathway may have contributed to the evolution of new developmental strategies.

Supplemental Data

Detailed Experimental Procedures and three supplemental figures are available at http://www.current-biology.com/cgi/content/full/15/7/643/DC1/.

Acknowledgments

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